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Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas

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ABSTRACT

Objective: Bile acids (BAs) facilitate fat absorption and may play a role in glucose and metabolism regulation, stimulating the secretion of gut hormones. The relative importance and mechanisms involved in BA-stimulated secretion of appetite and metabolism regulating hormones from the gut and pancreas is not well described and was the purpose of this study.

Methods: The effects of bile acids on the secretion of gut and pancreatic hormones was studied in rats and compared to the most well described nutritional secretagogue: glucose. The molecular mechanisms that underlie the secretion was studied by isolated perfused rat and mouse small intestine and pancreas preparations and supported by immunohistochemistry, expression analysis, and pharmacological studies.

Results: Bile acids robustly stimulate secretion of not only the incretin hormones, glucose-dependent insulinotropic peptide (GIP), and glucagon-like peptide-1 (GLP-1), but also glucagon and insulin *in vivo*, to levels comparable to those resulting from glucose stimulation. The mechanisms of GLP-1, neurotensin, and peptide YY (PYY) secretion was secondary to intestinal absorption and depended on activation of basolateral membrane Takeda G-protein receptor 5 (TGR5) receptors on the L-cells in the following order of potency: Lithocholic acid (LCA) >Deoxycholicacid (DCA)> Chenodeoxycholicacid (CDCA)> Cholic acid (CA). Thus BAs did not stimulate secretion of GLP-1 and PYY from perfused small intestine in TGR5 KO mice but stimulated robust responses in wild type littermates. TGR5 is not expressed on α-cells or β-cells, and BAs had no direct effects on glucagon or insulin secretion from the perfused pancreas.

Conclusion: BAs should be considered not only as fat emulsifiers but also as important regulators of appetite- and metabolism-regulating hormones by activation of basolateral intestinal TGR5.

Keywords Bile-acids; GLP-1; Neurotensin; Insulin; PYY; TGR5

1. INTRODUCTION

The gut is the source of several hormones with important effects on appetite (neurotensin (NT), glucagon-like peptide-1 (GLP-1), and polypeptide YY (PYY)) and glucose regulation (the incretin hormones, glucose-dependent insulinotropic peptide (GIP) and GLP-1) [7,16,29]. Activation of this system, therefore, represents a potential approach to treat type-2-diabetes and obesity, and this has generated considerable interest in understanding the molecular sensing mechanisms underlying the secretion of these hormones. Recent studies have indicated that BAs, in addition to their well-known role in fat absorption, may stimulate the secretion of a number of appetite- and metabolism-regulating peptide hormones from the gut and pancreas, including glucagon, GIP, GLP-1 insulin, and PYY [2,3,6,13,14,19,30,34,35,38,46,47], but the molecular sensing mechanisms involved are not well understood. The aim of this study was two-fold. First, we sought to evaluate the rat as a model for BA effects by examining the effects of BAs on the secretion of the most important appetite- and metabolism-regulating hormones from the gut and pancreas: ghrelin, GLP-1, NT, GIP, glucagon, insulin, and PYY and to compare the secretory responses to those elicited by glucose. Second, we sought to characterize the molecular sensing machinery

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behind the hormonal responses using a physiologically relevant model. The project relies on *in vivo* studies in rats as well as studies involving isolated, perfused preparations of the mouse and rat small intestine and the rat pancreas, allowing the secretory mechanisms to be studied in detail; these studies were supplemented with immunohistochemical and receptor pharmacology studies.

2. EXPERIMENTAL PROCEDURES

2.1. Chemicals

Test reagents were obtained as specified in supplementary methods.

2.2. Animal studies

Studies were conducted with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and the local ethical committee in accordance with the guidelines of Danish legislation governing animal experimentation (1987) and the National Institutes of Health (publication number 85-23).

2.3. Systemic effects of bile-acids in anesthetized rats — intraluminal stimulation

Male Wistar rats (~250 g) were obtained from Janvier (Saint Berthevin Cedex, France) and housed two per cage under standard conditions with ad libitum access to chow and water and left to acclimatize for at least one week before the study. Studies were carried out in different groups of rats on two occasions. On the day of study, rats were fasted 8 h prior to the study (1500 h) with access to water. Rats were anesthetized by a subcutaneous injection with Hypnorm/midazolam, then the lower part of the abdominal cavity was opened and a needle inserted into the inferior vena cava approximately 2 cm cranial to the iliac veins. A plastic tube was also inserted into the intestinal lumen approximately 8 cm below the pyloric sphincter. A basal blood sample was collected 5 min later and another immediately before stimulant administration. At time *0* min, rats ($n = 9$) were given 1.5 mL intraluminally of one of the following four solutions: 1) a complex bile acid mixture consisting of CA, GCA, TCA, GDCA, TDCA, DCA, CDCA, GCDCA, TCDCA; each 0.321 mM, 2) a mixture of UDCA and TUDCA, each at concentration of 1.404 mM, thus resulting in a similar total bile acid concentration as the complex bile acid mixture (2.808 mM), 3) D-glucose (50% (w/v): positive control), or 4) 0.9% NaCl (negative control). All stimulants were diluted in 0.9% NaCl. Rat body weights did not differ between treatment groups (305±5 g vs. 304±3 g, vs. 300±4 g vs. 295±5 g, $P > 0.05$ for all groups), and, on each study occasion, rats from the same cage received different treatments. Blood for hormone and blood glucose measurement was collected through the needle in vena cava (200 μL/time point; 1800 μL in total) into ice cold EDTA-coated tubes at time −5, 0, 2, 5, 10, and 30 min, and blood glucose was immediately measured by a handheld glucometer utilizing the gluco-oxidase method (Accu-chek Compact plus device, Roche, Mannheim, Germany). Samples for hormone analysis were centrifuged (1,650 × *g*, 4 °C, 10 min) to obtain plasma, which was transferred to fresh Eppendorf tubes and immediately frozen on dry ice. Samples were stored at −20 °C until analysis. In between sample collection, the needle was regularly flushed with isotonic salt water (~0.2 ml) to prevent clot formation. Samples were analyzed as described in the *biochemical measurement* section.

2.4. Isolated perfused rat and mouse small intestine and rat pancreas

Methods are described in more detail in the supplementary methods and elsewhere [8,9,12,24,44]. Male Wistar rats (~250 g) were housed as described above and acclimatized for a least a week. Heterozygous mice ($TGR5^{+/-}$) [46] were transferred to University of Copenhagen and bred to generate both $TGR5^{+/+}$ and $TGR5^{-/-}$ progeny. Animals were housed under same conditions as the rats. On the day of experiment, $TGR5^{+/+}$ or $TGR5^{-/-}$ mice (weight matched) or Wistar rats were anesthetized, and the abdominal cavity was opened. The upper half of the small intestine and the entire large intestine (for small intestine perfusions) or the entire intestine (for the pancreas perfusions) were carefully removed after tying off the supplying vasculature. Furthermore, for the pancreas perfusions, the spleen and stomach were removed, and the kidneys were excluded from perfusion by tying off the renal arteries. For the intestinal perfusions, a plastic tube was inserted into the lumen to allow administration of luminal stimulants. The small intestine (the distal half and the most proximal part connected to the pancreas) or the pancreas was perfused vascularly through the upper mesenteric artery or the abdominal aorta with warm (37 °C) modified Krebs ringer buffer gassed with 95% O_2 and 5% CO_2 (both v/v) until equilibrium, using a Uniper UP-100 perfusion system (Hugo Sachs; Harvard Apparatus, March-Hugstetten, Germany), and venous effluent were collected through a catheter in vena portae. As soon as the catheters were in place, the animals were killed by perforation of the diaphragm, and the preparation was allowed to equilibrate for approximately 30 min before sample collection Venous effluent was collected, immediately chilled on ice, and transferred to −20 °C within 30 min.

2.5. TGR5 expression in isolated murine α-, β-, δ- and L-cells

TGR5 expression in L-cell positive and L-cell negative epithelial cells were isolated from transgenic mice (GLU-Venus) expressing fluorescent proteins under the control of proglucagon promoter [36]. Pancreatic α-cells were isolated from GLU-Venus transgenic mice and pancreatic δ-cells from transgenic mice expressing YFP under the control of the somatostatin promoter as described previously [1]. The β-cells were isolated on the basis of their size, using forward and side scatter characteristics distinguishing them from other nonfluorescent islet cells in single cell preparations from mice with glucagon-driven Venus expression. TGR5 expression was determined by RT-PCR and expressed relative to beta-actin as described previously [36] and specified in supplementary methods.

2.6. Biochemical measurements

In vivo rat study: Plasma concentrations of peptide hormones were determined using a customized xMAP based multiplex-assay (Milliplex map rat metabolic hormone magnetic bead panel — metabolism multiplex assay, cat. no. RMHMAG-84 K, Millipore), for the following selected analytes: Amylin (Active), C-Peptide, Ghrelin (Active), GIP (Total), GLP-1 (total), Glucagon, IL-6, Insulin, Leptin, MCP-1, PP, PYY (Total), TNF-α. The assay is reported to have no significant cross-reactivity with other hormone tested. After the analytes were bound to the antibody-coupled beads, a biotinylated detection antibody was added, followed by incubation with streptavidin-phycoerythrin conjugate. Subsequently, the beads were subjected to a flow cytometry-based detection method using a Luminex laser-based analyzer (catalog no. Luminex 200, Luminex, Austin, TX 78727, USA). The manufacturer's instructions were closely followed. Reported intra- and inter-assay variation was 1−8% and 7−29%, respectively. The median fluorescent intensities from the eight calibrators were used to interpolate concentrations in plasma samples using 5-parameter logistic regression.

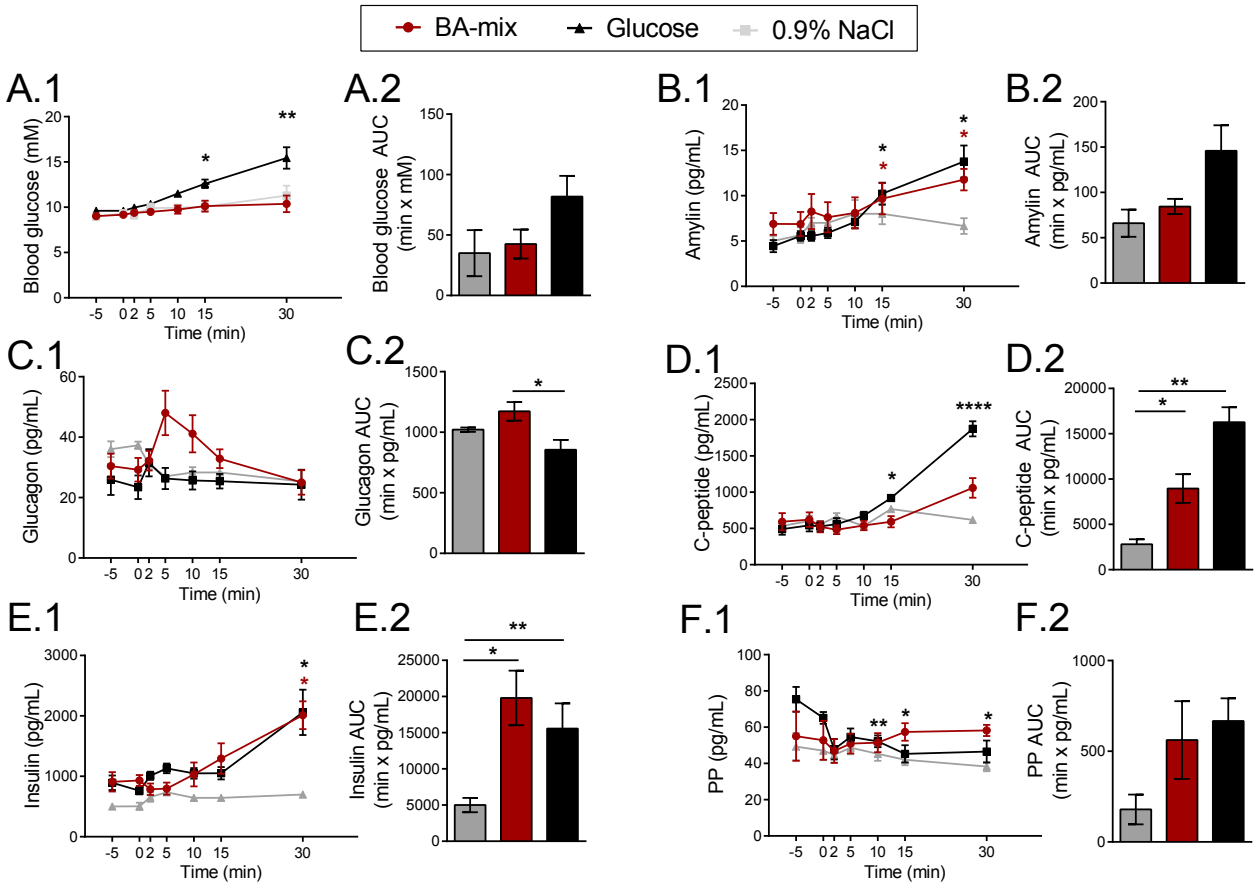

Figure 1: Effects of BAs on the secretion of pancreatic peptide hormones in anesthetized rats. Plasma mean values ± SEM are shown. A: Blood glucose, B: Amylin, C: Glucagon, D: C-peptide, E: Insulin, F: Pancreatic peptide (PP). * Red: Bile acid group, black: glucose group and grey: 0.9% NaCl group (neg. control). n = 9 (bile acid and glucose); n = 3 (0.9% NaCl). *P < 0.05, **P < 0.01 between respective baseline and response values or between treatment groups (AUCs).

2.7. Isolated perfused organs

Total bile acid concentrations in venous effluents were measured by an enzymatic assay relying on the ability of bile acids (in the presence of 3α-hydroxysteroid dehydrogenase) to reduce thio-NAD^+ to thio-NADH (detected) (Total Bile Acid Kit, Cat. no. STA-631, Cell Biolabs Inc. CA, USA). *Peptides from the isolated perfused mouse/rat intestine/pancreas* were quantified by use of extensively validated in-house radioimmunoassays (RIAs). GLP-1 concentrations were measured with assay 89390, employing an antibody specifically targeting the amidated C-terminus of the molecule, thus measuring total GLP-1 (both intact GLP-1 7-36amide, the primary metabolite GLP-1 9-36amide and other potential N-terminally truncated or extended isoforms) [32]. The amidated isoform of GLP-1 was targeted rather than the glycine extended isoform (GLP-1 7—37) because the rat predominantly stores and secretes amidated GLP-1 [22,45]. Rat PYY (total) was measured employing antibody T-4093, measuring both intact 1—36 and the primary metabolite PYY 3—36 [45]. NT (total) was measured using antibody 3D97, which targets N-terminal epitopes in the 1—8 sequence, thus targeting total NT [23]. Glucagon was measured with the C-terminally directed antibody 4305, which reacts with all bioactive forms of glucagon [31]. Insulin was measured using antibody 2006 which detects all bioactive forms of (murine) insulin [5]. Somatostatin was measured with a side-viewing antibody (assay 1758), thus detecting all bioactive peptide forms (SST-14 and SST-28) [17]. For all measurements, standard curves were prepared in perfusion buffer, which was shown to be devoid of matrix effects in control studies. Further assay details on the respective assays, including experimental detection limits, can be found elsewhere [25].

2.8. TGR5-induced cAMP production

COS-7 cells were grown in DMEM supplemented with 2 mM L-glutamine, 180 U/ml penicillin, 45 μg/ml streptomycin, and 10% (v/v) FBS according to protocols described previously [39]. 35,000 COS-7 cells per well were seeded in 96-well plates coated with poly-D-lysine and modified for either human or rat TGR5 expression using a transient calcium phosphate precipitation transfection procedure [21], using a pCMV6-XL5 or pCMV6-Entry vector, respectively (Cat. no. SC123312 and RN210451, OriGene, Technologies Inc., Rockville, MD). On the assay day, two days after transfection, growth medium was removed from the cells, and they were left to equilibrate in HBS buffer containing 1 mM IBMX for 30 min at 37 °C. Concentrations of different BAs or the TGR5 agonist RO6272296 were added to duplicate wells, and the cells were incubated for 30 min at 37 °C and subjected to *in vitro* HitHunter cAMP assay (based on enzyme fragment complementation, DiscoveRx) carried out according to the manufacturer's instructions. The TGR5 agonist was included in all runs to allow for data normalization between runs.

2.9. Immunohistochemistry

Control wild type C57BL/6 male mice and male Wistar rats (~300 g, Taconic, Ejby, Denmark) were killed and approximately 2 cm of the jejunum was collected. Tissue samples were fixed in formalin, paraffin embedded, sectioned, and processed using standard methods [51], The immuno-fluorescence staining protocol employed involved microwave treatment as previously described [52]. The double immunofluorescence staining was performed using (rabbit) anti-GLP-1 (ab22625, Abcam® 330 Cambridge Science Park, Cambridge, CB4 0FL, UK) and (goat) anti-TGR5 (Cat. no. SC-48687, Santa Cruz Biotechnology, Inc. Dallas, TX) diluted 1:50 in 10% (v/v) FBS in PBS. Sections were incubated overnight at 4 °C, washed thoroughly with PBS, and then double labeled with (donkey) anti-rabbit IgG (H + L): Alexa Fluor® 546 conjugate (Cat. no. A10040, ThermoFischer Scientific, Slangerup, Denmark) and (donkey) anti-goat IgG (H + L): Alexa Fluor® 488 conjugate (Cat. no. A11055, ThermoFischer Scientific) after 30 min incubation. Cell nuclei were stained with DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride). Fluorescence imaging was performed using a Zeiss confocal 510 microscope equipped with a water 63x/1.0 Plan-Apochromat objective and Zen software (Carl Zeiss, Oberkochen, Germany). In a separate set of experiments, the specificity of the TGR5 antibody applied above as well as another TGR5 antibody (Cat. no. LS-A1936, LifeSpan BioSciences, Inc., Seattle, WA), was validated by transfecting HEK293 with cDNA clones containing mouse or rat (Myc-DKK-tagged) TGR5 transcript (Cat. no. MR227683 and RR 210451) or with mock vector. Antibody SC-48687 strongly reacted with cells transfected with either mouse and rat TGR5 cDNA (identified by co-expression of c-Myc), whereas the other TGR5 antibody (LS-A1936) did not react with TGR5 transfected cells and were therefore judged unsuitable for further characterization (data shown in Supplementary Fig. 1A). To further validate the specificity of the SC-48687 antibody, we applied the same protocol as described above on small intestinal tissue collected from a TGR5 KO mouse or wild type littermate. Staining was confirmed in the tissue from the wild type littermate but not the TRG5 KO mouse (Supplementary Fig. 1B). IBAT staining was performed on the same tissue by similar methods using an antibody from Aviva Systems Biology (Cat. no. OAEB00210, San Diego, CA).

2.10. Data presentation and statistical analysis

In vivo data are presented as absolute mean values ± SEM and as baseline-corrected (calculated as the mean of −5 and 0 min concentrations) area under the curve (AUC) values to compare overall effects during the course of the study between treatments. Perfusion data are presented as absolute mean values ± 1SEM. *TGR5* pharmacology data are presented normalized to the maximal [cAMP] response obtained by the TGR5 agonist RO6272296. Statistical significance of responses was tested by: *In vivo* responses: One-way ANOVA followed by the Tukey post hoc test where appropriate, either testing the individual time points against each of the stimuli's

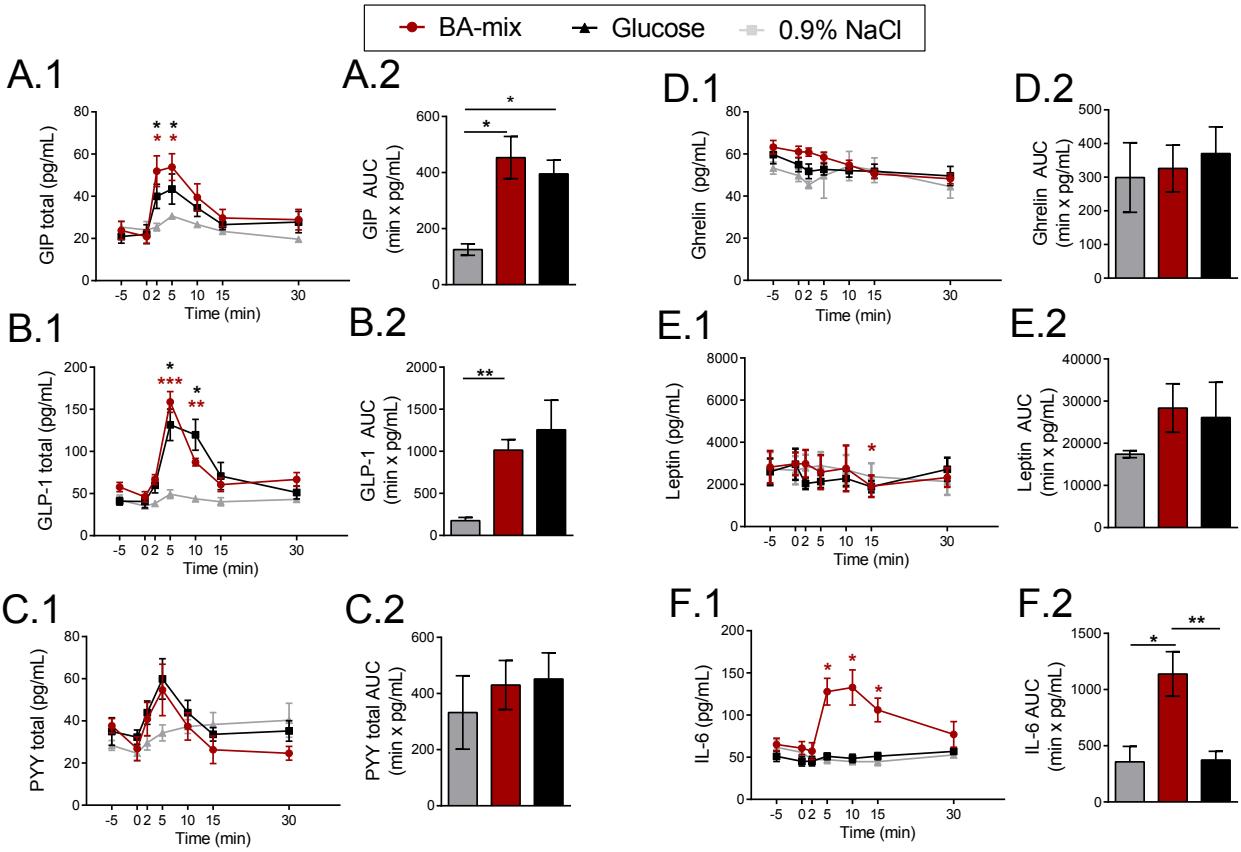

Figure 2: Effects of BAs on the secretion of gut-peptide hormones in anesthetized rats. Plasma mean values ± SEM are shown. A: GIP (total), B: GLP-1 (total), C: PYY (total), D: Ghrelin, E: Leptin, F: IL-6. Red: Bile acid group, black: glucose group and grey: 0.9% NaCl group (neg. control). n = 9 (bile acid and glucose); n = 3 (0.9% NaCl). *P < 0.05, **P < 0.01, ***P < 0.001 between respective baseline and response values.

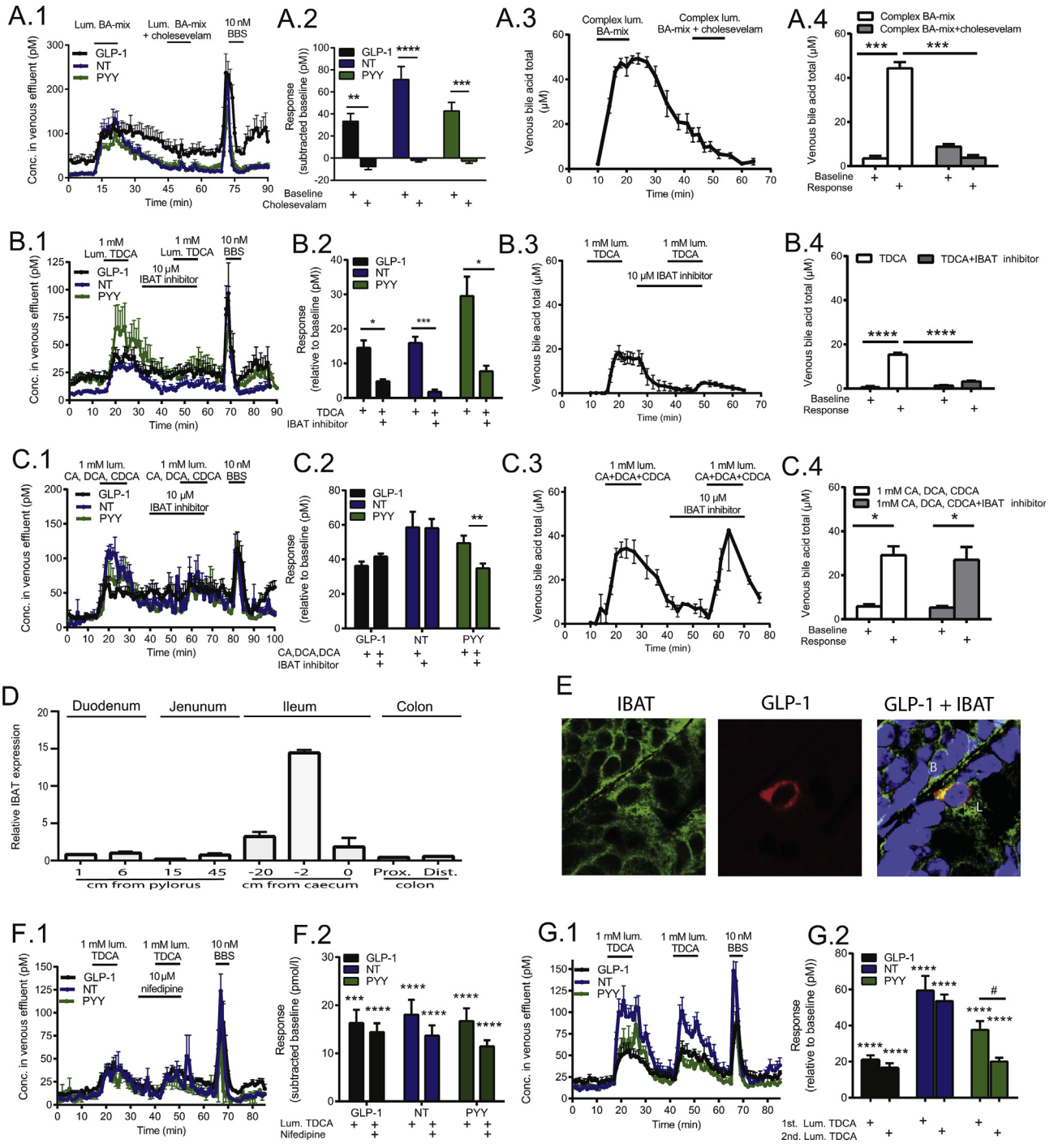

Figure 3: Bile acid-stimulated GLP-1, NT and PYY secretion depends on intestinal absorption, but electrogenic-absorption through Ileal-bile acid-transporter (IBAT) does contribute to the response. Data are shown as means ± 1 SEM. *A1-4:* Effects of luminal instillation of a complex BA-mix +/− the bile acid sequestrant cholevelam on GLP-1, NT and PYY secretion (A.1.2) and bile acid absorption (A.3.4), *n=6. B1-4:* Effects of luminal TDCA +/− luminal IBAT inhibitor on GLP-1, NT, and PYY secretion (B1,2) and TDCA-absorption (B3,4), *n=6. C1-4:* Effects on an un-conjugated BA-mixture (CA, DCA, and CDCA) +/− IBAT inhibitor on GLP-1, NT, and PYY secretion (C1,2) and BA absorption (C3,4), *n=6. D:* IBAT expression down the rat small intestine, *n=4, E:* IBAT and GLP-1 expression in rat intestinal tissue. *F1,2:* Effects of TDCA +/− nifedipine (a voltage-gated calcium-channel blocker) on GLP-1, NT, and PYY secretion, *n=6. G1,2:* Effects of luminal TDCA on GLP-1 and PYY secretion (control experiment), $n = 6$. In all perfusion experiments, bombesin (BBS) was administered in the end of the experiment and used as positive control. $^{*/\#}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

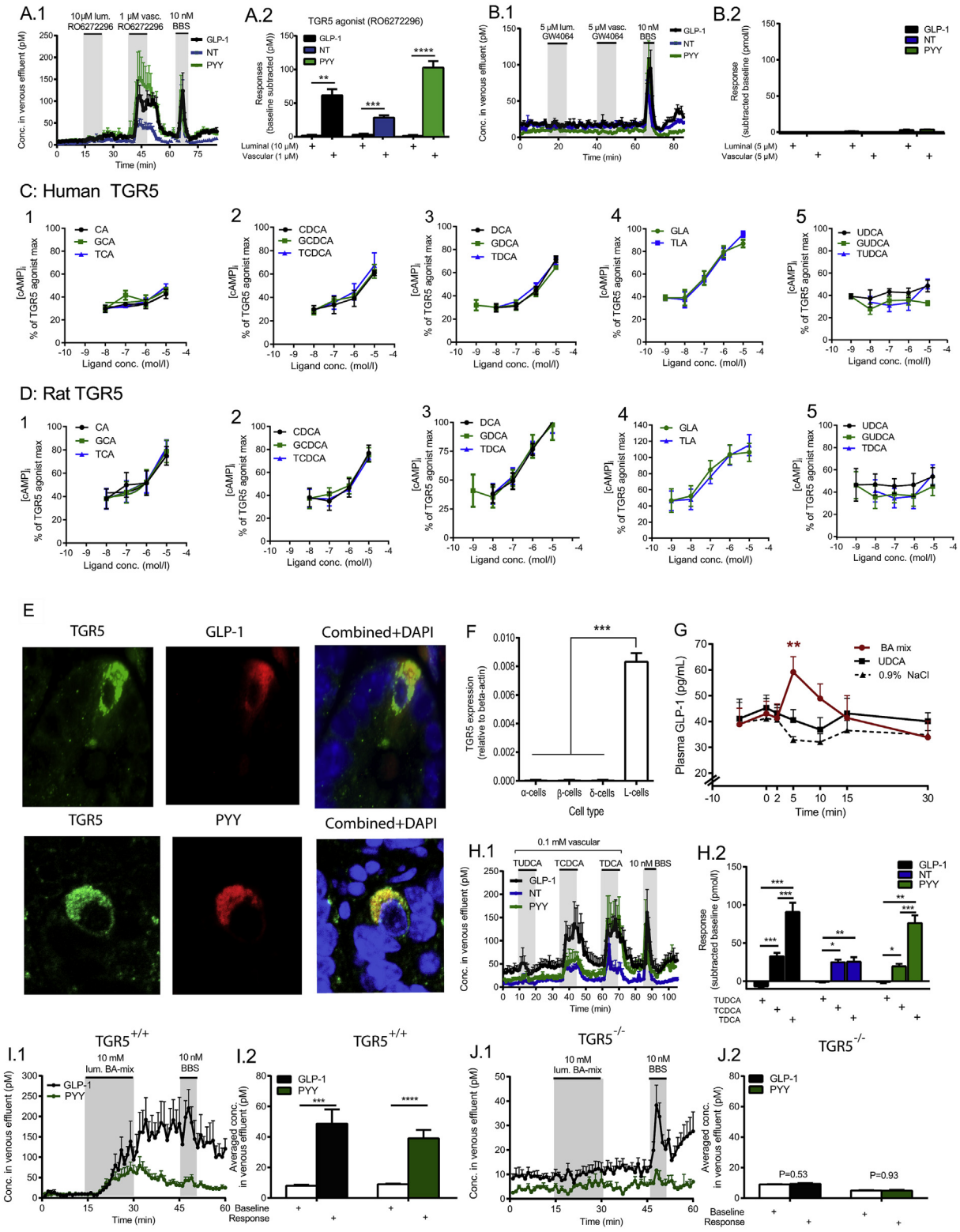

Figure 4: Bile acids stimulate GLP-1, NT, and PYY secretion by activation of basolateral, but not luminal, TGR5 receptors. Data are shown as means ± 1 SEM. All X-Y plot data with exception of C, D, F, I and J are from isolated perfused rat small intestine. *A1,2:* Effects of luminal and vascular administration of a poorly absorbable TGR5 agonist on GLP-1, NT and PYY secretion, *n=6*. *B1,2*: Effects of luminal and vascular administration of GW4064 (a FXR agonist) on GLP-1, NT and PYY secretion, *n=6*. *C,D*: Activation of TGR5 in response to different bile-acids in cells transfected with either the human TGR5 receptor (C) or rat TGR5 receptor (D). *E:* Co-localization of TGR5 and GLP-1/PYY in rat small intestine. *F*: Expression of TGR5 in isolated pancreatic α-, β- or δ-cells or intestinal L-cells from the mouse, *n=3*. *G*: Plasma GLP-1 (total) concentrations in response to a complex BA-mix (*n=6*), match total conc. of UDCA (*n=8*) or 0.9%NaCl (neg. control, *n* = 3). *H1,2:* Effects of vascular TUDCA, TCDCA or TDCA on GLP-1, NT and PYY secretion, *n=6*. I and J: Effects of a complex BA-mix on the secretion of GLP-1 and PYY from isolated perfused mouse small intestine. I: WT mice, J: TGR5 KO mice, n = 6. In all perfusion experiments, bombesin (BBS) was administered in the end of the experiment and used as positive control. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

own baseline (average of −5 and 0 min) or testing AUCs against each other. Perfusion responses were assessed by comparing averaged concentrations during the stimulation period (10 consecutive 1-min observations) with mean basal levels over a similar duration: immediately before the stimulation (5 consecutive 1-min observations) and at the end of the following equilibrium period (also 5 consecutive 1-min observations). Significance was assessed by One-way-ANOVA followed by Tukey post hoc. For the remaining data, unpaired or paired two-sided t-test was used to assess differences between two groups, as indicated. For all tests, threshold for significance was set to $P = 0.05$.

3. RESULTS

3.1. Intra-luminal delivery of bile acids stimulates secretion of numerous appetite- and metabolism-regulating peptides in anesthetized rats

Blood glucose was unaltered by an intra-luminally administered BA-mix (cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) both in free form and in their glycine- and taurine-conjugated forms, 1 mM each) ($P > 0.05$, Figure 1A) but increased after intra-luminal glucose treatment (baseline: 9.6 ± 0.2 mM, peak: 15.5 ± 1.2, $P < 0.001$). Glucose as well as the BA-mix stimulated amylin secretion 15 and 30 min after administration ($P < 0.05$, Figure 1B). Glucagon secretion increased after BA-mix administration at time point 5 min ($P < 0.05$, Figure 1.C1) and net $AUC_{0-15min}$ increased ($P_{NaCl\ vs.\ BA\text{-}mix} = 0.07$, Figure 1.C2). Both glucose and BAs stimulated plasma C-peptide and insulin secretion at the later time points (Figure 1.D,E). GIP and GLP-1 secretion was robustly stimulated by the bile acid mix (2−4 fold) at 5 min ($P < 0.05$ compared to baseline, $n = 9$ Figure 2A,B) and returned to baseline at 15 min. Similar responses were observed after intra-duodenal glucose, and neither peak-value nor AUCs differed between treatments ($P_{GIP,\ glucose\ vs.\ BA\text{-}mix\ AUC's} = 0.78$, $P_{GLP\text{-}1,\ glucose\ vs.\ BA\text{-}mix\ AUC's} = 0.77$, $n = 9$, Figure 2A,B). Glucose and BAs both tended to increase PYY secretion (Figure 2C). For all measured peptides, plasma concentrations remained unchanged at all time points in the isotonic saline control group ($P > 0.05$, Figures 1 and 2).

3.2. Molecular mechanisms of bile-acid stimulated secretion of GLP-1, NT and PYY

3.2.1. Effects of bile acids and a bile acid sequestrant

Since GLP-1 and PYY secretion was stimulated by BAs *in vivo*, we next investigated the molecular mechanisms underlying these (as well as NT) responses using the isolated, perfused rat small intestine. Intra-luminal instillation of a BA mixture (CA, DCA, CDCA in both un-conjugated and glycine- and taurine-conjugated forms) increased secretion of GLP-1, PYY and NT to 4−8 times the basal secretion, and this was prevented by cholesevelam (Figure 3.A1−2, n = 6). Cholesevelam is a BA sequestrant, which binds to BAs and reduces their absorption (Figure 3A3−4). In order to investigate whether the lack of gut hormone secretion in the presence of this BA-sequestrant (Figure 3A1−2) was due to reduced availability of free BAs to stimulate luminally exposed receptors or to reduced absorption and reduced exposure of basolateral BA (Figure 3D,G), we next tested the effects of inhibiting the predominant transporter for conjugated bile acids, the ileal-bile acid transporter (IBAT).

3.2.2. Role of the ileal-bile acid transporter (IBAT)

Transporter inhibition attenuated both TDCA absorption and hormone secretion (Figure 3 B1−4, *n=6*). In a separate line of experiments, we tested the effect of the IBAT inhibitor together with intra-luminal infusion of a mixture of unconjugated BAs (CA, DCA, and CDCA: each 1 mM), which are more lipophilic than the conjugated-BAs (and should, therefore, be absorbed by IBAT-*independent* diffusion over the intestinal epithelium). In this case, the inhibitor had no effects on either the BA absorption or the GLP-1 and NT responses, (although PYY secretion was slightly reduced) ($P < 0.01$, Figure 3.C1−4, *n=6*). As IBAT couples BA uptake to the co-uptake of sodium, activity of this transporter could (if present on the respective enteroendocrine cells), in itself stimulate peptide secretion by depolarizing the L-cell, as is the case for glucose-stimulated GLP-1 and NT secretion (through the sodium-glucose transporter 1 [11,22−24,33,37]). In the rat, IBAT was predominately expressed in the ileum (Figure 3D) and co-localized with GLP-1 (Figure 3E), but the effect of IBAT activity on TDCA-stimulated GLP-1, NT, or PYY secretion from the perfused rat intestine does not appear to involve cell depolarization since inhibition of L-type voltage-gated calcium channels (by nifedipine) had no effect on the secretory response (Figure 3F.1,2). In separate control studies, intra-luminal administration of TDCA robustly stimulated the secretion of GLP-1, NT, and PYY and in case of GLP-1 and NT to a comparable extent when applied twice in the same experiment (Figure 3 F1, 2, $n = 6$), confirming that the attenuation of TDCA-stimulated secretion in presence of the IBAT inhibitor was a specific event.

3.2.3. The role of farnesoid-X-activated receptor or G protein-coupled bile acid receptor 1 (GPBAR1/TGR5)

We next investigated whether the secretory response to BAs could be explained by activation of the BA-sensitive receptors FXR or TGR5. Intra-arterial, but not intra-luminal, application of a poorly absorbable TGR5 agonist [48] robustly stimulated GLP-1, NT and PYY secretion (Figure 4A.1,2), while application of a specific FXR agonist had no effect whether applied from the luminal or vascular route (Figure 4B1,2). In COS-7 cells transiently transfected with either the human or rat TGR5 (Figure 4C,D), a strong activating signal was observed with pharmacologically relevant BA doses ($EC_{50} < 1$ μM) in following order of potency: LCA > DCA > CDCA acid for both receptors. Comparable efficacy and potency were observed for free, taurine- and glycine-conjugated forms of the respective BAs. In contrast, UDCA (free or taurine- and glycine-conjugated) did not activate human or rat TGR5 in concentrations up to 10 μM, while CA (free as well as glycine- and taurine-conjugated forms) was a weak agonist for the rat but not the human receptor (Figure 4C1−5, D1−5). Additional pharmacological properties of the tested BAs (Hill slope, time to peak, EC_{50}-value and percentage efficacy compared to maximal cAMP-response resulting from TGR5-agonist stimulation) are provided in Supplementary Table 1. As we found TGR5 to be expressed by isolated primary mouse L-cells and to co-localize with GLP-1 and PYY in rat small intestine (Figure 4E,F), our data, therefore, indicate that BA-stimulated secretion is dependent on direct activation of basolaterally located TGR5 receptors expressed by the N- or L-cell. Intra-luminal UDCA (which does not activate TGR5) *in vivo* did not change GLP-1 secretion, whereas a complex BA mixture (CA, DCA, and CDCA in un-conjugated and glycine- and taurine-conjugated forms) with the same total BA concentration resulted in a pronounced increase (Figure 4G). Furthermore, intra-arterial TUDCA (0.01 mM) neither stimulated GLP-1, NT nor PYY secretion from isolated perfused rat small intestine, in contrast to the TGR5-activating BAs TCDCA and TDCA, both of which robustly stimulated secretion when applied at the same concentration ($P < 0.001$, $n = 6$, Figure 4H1,2. To further demonstrate the role of the TGR5 receptor, we also studied isolated perfused small intestine from TGR5 knockout (KO) mice [46]. Here,

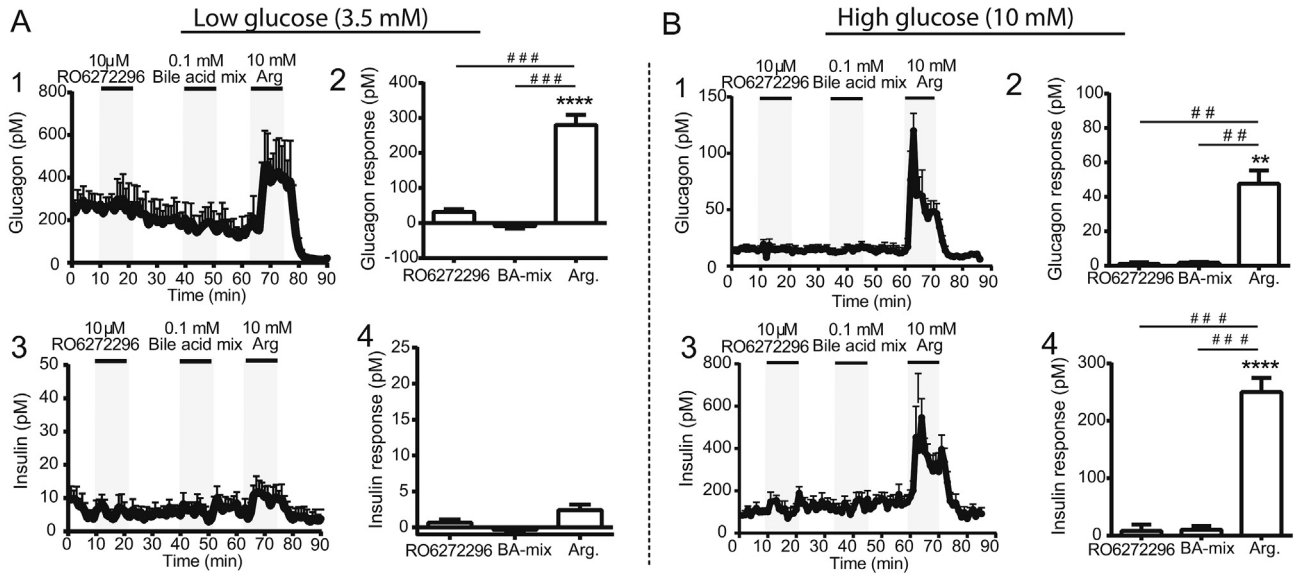

Figure 5: TGR5 activation or bile acids has no direct effects on glucagon or insulin secretion. Data are shown as means ? 1 SEM. All data are from isolated perfused rat pancreas. A. Glucagon (1,2) and insulin (3,4) secretion at low glucose (3.5 mmol/L) in response to the TGR5 agonist RO9272296 or a complex BA-mix. B: Glucagon (1,2) and insulin (3,4) secretion at high glucose (10 mmol/L) in response to the TGR5 agonist RO9272296 or a complex BA-mix. L-arginine (Arg) was included at the end of all experiment and used a positive control. n = 6 for all experiments. *P < 0.05, **/##P < 0.01, ***/###P < 0.001, ****P < 0.0001. Stars indicate significance compared to baseline and hashes indicate significance between treatments.

intra-luminal administration of bile acids (same complex mixture as above) had no effects on GLP-1 and PYY secretion, whereas a robust hormone response was seen in wild-type littermates (Figure 4I,J).

3.3. No direct effects of bile acids on the secretion of pancreatic hormones

To investigate whether the BA-stimulated insulin response and the tendency to stimulated glucagon secretion observed *in vivo* resulted from direct effects of BAs on the pancreas, we studied the direct effects of BAs and direct TGR5 activation in the isolated perfused rat pancreas, using the same BA-mix and TGR5 agonist used *in vivo* and in the intestine perfusions. Neither BAs nor the TGR5 agonist (even at a 10-fold higher dose than that which robustly stimulated GLP-1, NT and PYY secretion in the gut) had any effect on glucagon or insulin secretion, whether at low (3.5 mM) or high (10 mM) glucose levels (Figure 5A—D). consistent with our observation that neither pancreatic α- nor β-cells isolated from mice express TGR5 (Figure 4F). Glucose had the expected effects on the secretion of these peptides: raising the glucose concentration from 3.5 to 10 mM inhibited glucagon secretion by a factor of approx. 15 and stimulated insulin secretion by a factor of approx. 10, while L-arginine (pos. control) also robustly stimulated the secretion of both hormones.

4. DISCUSSION

In humans, BAs have been identified as stimuli for glucagon, GLP-1, insulin, NT, and PYY secretion [2,3,13,30,38], but a detailed analysis of the physiological sensing mechanisms involved has not been carried out, and this was the purpose of our study. Because of their chemical nature, unspecific effects of BAs could potentially confound interpretation of results, especially when used in high concentrations. Since such studies cannot easily be carried out in humans, we turned to rats and first investigated the effects of intraluminal BA stimulation (a complex mixture) on a number of appetite- and metabolism-regulating peptide hormones in anesthetized animals. The BAs were administered in a dose similar to that reached intra-luminally in the proximal small intestine in humans after meal intake, i.e. 4—12 mM depending on meal composition [41,49] and well above the critical micellar concentration 1—2 mM [15]. However, whether the composition of the applied BA mix in our study is representative for the postprandial intraluminal composition in humans (or rats) is difficult to assess. This is because most data on the composition of BAs in humans have been collected from samples obtained from a peripheral vein. Little is known about how closely this reflects the composition of BAs originally secreted into the intestinal lumen, because intestinal reabsorption and liver retention may differ between BAs and because of differences in the microbial activity in the gut which dehydroxylates the primary Bas. In the anesthetized rat, intraluminal administration of the complex BA-mix (9 mM in total) robustly stimulated the secretion of GIP, GLP-1, C-peptide and insulin, mirroring the effects of BAs in humans [2,3,13,38]. Remarkably, the secretory responses were of comparable magnitude to those resulting from intraluminal glucose administration (a powerful stimulus for most of these peptides [18]). For PYY, the half hour sample collection period may have been too short to allow the stimulants to reach all of the more distally located PYY.

To study the secretory pathways directly involved in BA-stimulated gut hormone secretion, we used the isolated perfused rodent small intestine. The benefit of this model is that it allows a high level of experimental control (*e.g.* it is possible to discriminate between responses caused by luminal or vascular activation of secretory sensors), high time resolution (secretion is followed minute by minute), and application of drugs that may have been hazardous or lethal *in vivo,* as reviewed [43]. Additionally, the natural anatomical arrangement of the cells (including polarization) and the vascular perfusion (and therefore convective drag of absorbed nutrients and secreted hormones) are preserved [43], allowing studies of the full dynamics of absorption and secretion. Thus, conclusions drawn from results obtained using this model are likely to be physiologically relevant.

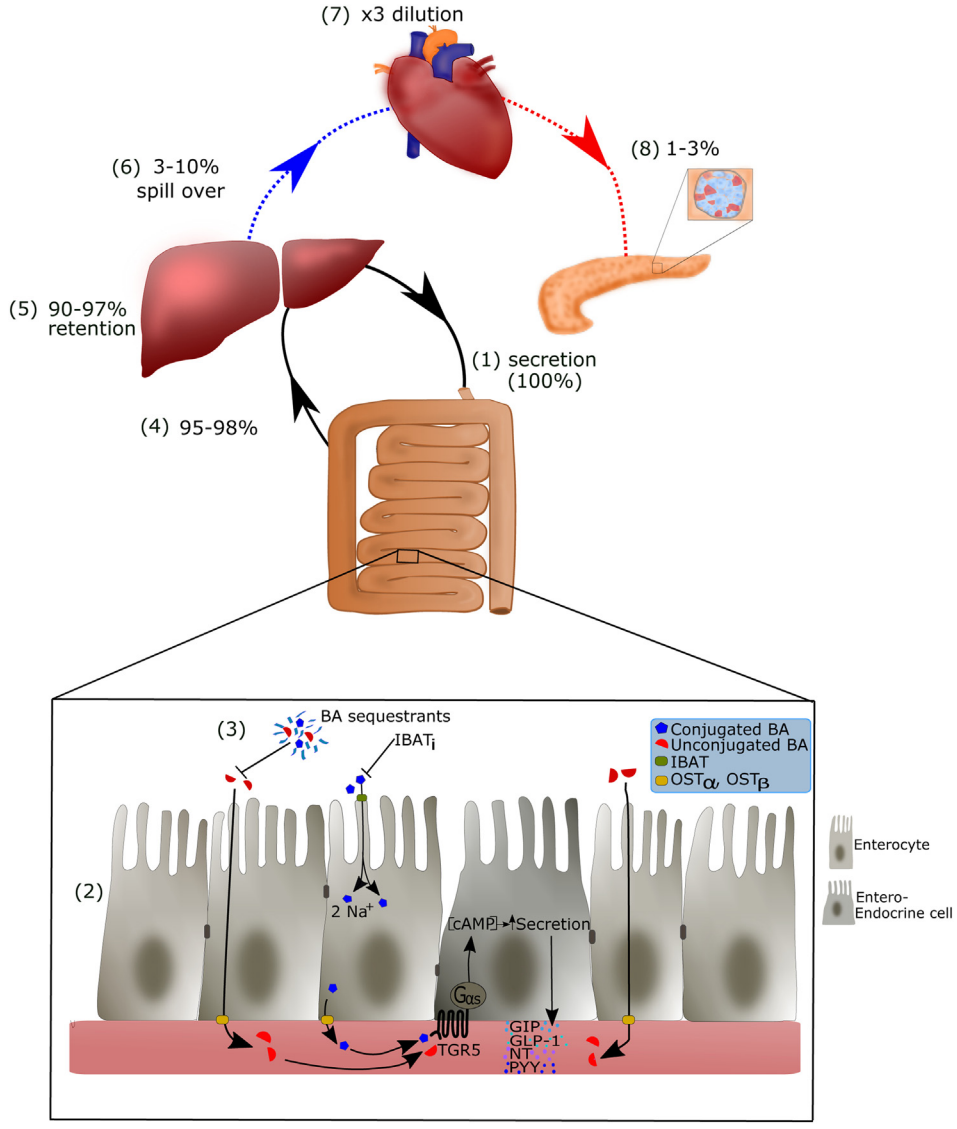

Figure 6: Proposed model of bile acid stimulated secretion of appetite and metabolism regulating hormones. (1) Food intake, and in particular fat consumption, stimulates the secretion of bile acids into the upper small intestine by CCK-mediated contraction of the gallbladder. (2) In addition to their well-known role in facilitating fat absorption (by micelle formation) bile acids activate TGR5 receptors which are located at the basolateral membranes of the enterocytes and therefore are activated secondary to bile acid (BA) absorption. Upon activation, the secretion of GIP, GLP-1, NT, and PYY is stimulated. Conjugated bile acids are absorbed through the secondary active transporter ileal-bile acid transporter (IBAT) which is predominantly expressed in the lower part of the small intestine, whereas unconjugated bile acids (which are more lipophilic) spontaneously cross the intestinal mucosal layer. (3) Eliminating bile acid absorption by BA-sequestrants (which cross bind both conjugated and unconjugated BAs into large unabsorbable complexes) or by direct IBAT inhibition (which attenuates the absorption of conjugated BAs) therefore eliminates BA-stimulated gut hormone secretion. (4) Collectively, the absorption mechanisms results in a very efficient BA absorption so about 95% of the secreted bile acids are returned to the liver through the enterohepatic circulation. (5) The majority of the returned bile acids are extracted by the liver (where they are reconjugated and rehydroxylated, allowing the same pool of bile acids to be secreted several times during the day), (6) Only 3–10% pass the liver and eventually ends up in the systemic circulation, (7) The pool is further diluted with a factor of about three since the hepatic return constitutes about 1/3 of the total venous return. (8) Therefore, only a small fraction of the secreted bile acid makes it to the systemic circulation.

Similar to the *in vivo* study, intra-luminal BA administration resulted in robust GLP-1, NT, and PYY responses, confirming *in vitro* studies where a variety of BAs have been shown to stimulate GLP-1 secretion from the GLP-1 producing cell lines GLUTag and STC-1, from primary murine cell cultures, and from mouse gut tissue mounted in Ussing chambers [6,19,34]. In our perfused gut model, we are able to demonstrate that peptide secretion depends on the absorption of BAs, since BA-sequestrants (cholesevelam) or pharmacological inhibition of the transporter responsible for conjugated BA uptake from the intestinal lumen, IBAT, eliminated the secretory responses. In contrast, the secretory responses to a mixture of unconjugated BAs (which are absorbed by IBAT-independent diffusion over the intestinal epithelium) were unaffected by IBAT inhibition. Consistent with this, IBAT inhibition attenuated conjugated BAs stimulated GLP-1 secretion from mouse epithelium mounted in Ussing chambers [6]. Although L-cells have been shown to express IBAT [6] and we here demonstrate co-localization (by immunohistochemistry) of this transporter with GLP-1, the secretory response to luminal (conjugated) BAs did not appear to depend on cellular depolarization resulting from coupled sodium transport, since inhibition of voltage-gated calcium channels (by

nifedipine) had no effect on the secretory response to conjugated BAs (whereas the same dose of nifedipine has been shown to eliminate glucose-stimulated GLP-1 and NT secretion [23,24]). This is consistent with our previous observation with regards to BA-stimulated GLP-1 secretion from mouse gut tissue mounted in Ussing chambers [6]. We therefore next investigated whether stimulated secretion relied on activation of BA-sensitive receptors. BAs have previously [6,46] been shown to activate the surface receptor TGR5 [20] and the nuclear receptor FXR, with the latter being effectively activated by CDCA, TCA, TCDCA, TDCA (but not tauro-muricholic acid) [28,50]. In our perfused rat intestine model, application of a specific FXR-agonist (GW4064) from the luminal or vascular side (at concentrations far above the reported EC_{50} of 15 nM [4]) had no effect on peptide secretion, indicating that activation of FXR is not acutely involved in the secretory response. Rather, this nuclear receptor may play a role for the beneficial (transcriptional) adaptations resulting from gastric bypass operations in mice [40]. In contrast, administration of a poorly-absorbable TGR5 agonist [48] to the perfused rat intestine from the vascular, but not the luminal, side of the preparation robustly stimulated the secretion of GLP-1, NT, and PYY. This is consistent with a study by Ullmer C et al. who showed that systemic rather than luminal activation of TGR5 stimulated GLP-1 and PYY secretion in mice, since intravenous but not oral administration of the same TGR5 agonist used here stimulated secretion [48]. By immunohistochemical and pharmacological studies, we showed that BAs appear to stimulate secretion of these peptides by direct activation of TGR5 at the level on the L-cell, because the receptor is co-localized with GLP-1 on the basolateral side of the cell and is activated (both human and rat versions of the receptor) by a variety of BAs in the following order of potency: LA $>$ DCA $>$ CDCA, irrespectively of conjugation. Consistent with this, UDCA and its glycine- and taurine-conjugated isoforms neither activated the receptor nor stimulated GLP-1 secretion. Since a TGR5 antagonist, which could provide further evidence for the role of this receptor is not available, we used TGR5 KO mice [46] and wild type littermates for gut perfusion studies [12]. In line with our observations from the rat and two studies using other experimental models [6,46], TGR5 deficiency resulted in elimination of BA-stimulated GLP-1 and PYY secretion.

BAs or selective TGR5 agonists have been suggested to directly stimulate glucagon and insulin secretion from isolated human and rodent islets [10,26,27]. However, although we observed stimulatory effects of BAs on insulin secretion *in vivo,* these seemed to be indirect, as the same complex BA-mixture that gave rise to robust secretion of gut hormones had no effect on secretion of glucagon or insulin from the perfused rat pancreas. Furthermore, TGR5 was not expressed in primary α- or β-cells and a TGR5 agonist (which stimulated secretion of GLP-1, NT, and PYY from the perfused intestine) had no effect on endocrine hormone secretion from the perfused pancreas. Therefore, the late increase in insulin secretion could be a result of BA-stimulated secretion of GIP and GLP-1, which could be tested in experiments involving blockade of the relevant hormone receptors (with GIP and GLP-1 receptor antagonists, *e.g.* the truncated GIP isoform $3\text{-}30_{NH2}$ recently showed to be a competitive antagonist for the rat GIP receptor [42] and the widely used GLP-1 receptor antagonist, exendin 9-39).

In contrast, glucagon and insulin secretion was very sensitive to changes in the glucose concentration in the perfusate and responded to our positive control (L-arginine), confirming that the preparation was functional and responds appropriately to physiological stimuli. BA-stimulated secretion of insulin, therefore, appears to be mediated indirectly and, although this remains to be clarified, potential mediators could include GIP, GLP-1, fibroblast growth factor (FGF19/and/or 21). It therefore appears that BAs stimulate the secretion of a number of gut hormones by activation of TGR5 located on the basolateral side of the enterocytes, whereas the effects of BAs on glucagon and insulin secretion is indirect. A detailed model for the molecular mechanisms underlying BA-stimulated secretion of gut hormones is provided in Figure 6.

5. CONCLUSION

Our study shows that BAs that activate TGR5 have marked effects on the secretion of appetite and metabolism-regulating hormones and, therefore, in addition to their role as fat emulsifiers, should be regarded as important regulators of blood glucose and metabolism. Given the strong stimulatory effect on GIP, GLP-1, NT, and PYY secretion, the mechanisms of BA-stimulated secretion, here demonstrated to depend on absorption and TGR5 activation, may represent appealing targets for development of drugs for treatment of obesity and type-2-diabetes.

AUTHOR CONTRIBUTIONS

REK and JJH created the overall study design. REK, NJWA, OL, SLJ, EBM, CFD, FR, FMR, RA, BH, MMR, and JJH designed details and interpreted results. REK, NJWA, OL, SLJ, EBM, RA, FR, and BH performed experiments. REK, NJWA, and RA analyzed data and prepared figures. REK drafted the manuscript. REK, NJWA, OL, SLJ, EBM, CFD, FR, FMR, RA, BH, MMR, and JJH edited the manuscript and contributed with intellectual content and approved the final version.

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CONFLICTS OF INTEREST

The authors have nothing of relevance to this study to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.03.007.

REFERENCES

[1] Adriaenssens, A.E., Svendsen, B., Lam, B.Y., Yeo, G.S., Holst, J.J., Reimann, F., et al., 2016. Transcriptomic profiling of pancreatic alpha, beta and delta cell populations identifies delta cells as a principal target for ghrelin in mouse islets. Diabetologia 59:2156–2165.

[2] Adrian, T.E., Ballantyne, G.H., Longo, W.E., Bilchik, A.J., Graham, S., Basson, M.D., et al., 1993. Deoxycholate is an important releaser of peptide YY and enteroglucagon from the human colon. Gut 34:1219–1224.

[3] Adrian, T.E., Gariballa, S., Parekh, K.A., Thomas, S.A., Saadi, H., Kaabi, J., et al., 2012. Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers. Diabetologia 55:2343–2347.

[4] Akwabi-Ameyaw, A., Bass, J.Y., Caldwell, R.D., Caravella, J.A., Chen, L., Creech, K.L., et al., 2008. Conformationally constrained farnesoid X receptor (FXR) agonists: naphthoic acid-based analogs of GW 4064. Bioorganic & Medicinal Chemistry Letters 18:4339–4343.

[5] Brand, C.L., Jorgensen, P.N., Knigge, U., Warberg, J., Svendsen, I., Kristensen, J.S., et al., 1995. Role of glucagon in maintenance of euglycemia in fed and fasted rats. The American Journal of Physiology 269:E469–E477.

[6] Brighton, C.A., Rievaj, J., Kuhre, R.E., Glass, L.L., Schoonjans, K., Holst, J.J., et al., 2015. Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G protein-coupled bile acid receptors. Endocrinology 156: 3961–3970.

[7] Campbell Jonathan, E., Drucker Daniel, J., 2013. Pharmacology, physiology, and mechanisms of incretin hormone action. Cell Metabolism 17: 819–837.

[8] Christiansen, C.B., Svendsen, B., Holst, J.J., 2015. The VGF-Derived neuropeptide TLQP-21 shows No impact on hormone secretion in the isolated perfused rat pancreas. Hormone and metabolic research Hormon und Stoffwechselforschung Hormones et metabolisme 47:537–543.

[9] de Heer, J., Holst, J.J., 2007. Sulfonylurea compounds uncouple the glucose dependence of the insulinotropic effect of glucagon-like peptide 1. Diabetes 56:438–443.

[10] Dufer, M., Horth, K., Wagner, R., Schittenhelm, B., Prowald, S., Wagner, T.F., et al., 2012. Bile acids acutely stimulate insulin secretion of mouse beta-cells via farnesoid X receptor activation and K(ATP) channel inhibition. Diabetes 61: 1479–1489.

[11] Gorboulev, V., Schürmann, A., Vallon, V., Kipp, H., Jaschke, A., Klessen, D., et al., 2012. Na+-d-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. Diabetes 61:187–196.

[12] Grunddal, K.V., Ratner, C.F., Svendsen, B., Sommer, F., Engelstoft, M.S., Madsen, A.N., et al., 2016. Neurotensin is coexpressed, coreleased, and acts together with GLP-1 and PYY in enteroendocrine control of metabolism. Endocrinology 157:176–194.

[13] Hansen, M., Scheltema, M.J., Sonne, D.P., Hansen, J.S., Sperling, M., Rehfeld, J.F., et al., 2016. Effect of chenodeoxycholic acid and the bile acid sequestrant colesevelam on glucagon-like peptide-1 secretion. Diabetes, Obesity & Metabolism 18:571–580.

[14] Harach, T., Pols, T.W., Nomura, M., Maida, A., Watanabe, M., Auwerx, J., et al., 2012. TGR5 potentiates GLP-1 secretion in response to anionic exchange resins. Scientific Reports 2:430.

[15] Hofmann, A.F., 1963. The function of bile salts in fat absorption. the solvent properties of dilute micellar solutions of conjugated bile salts. The Biochemical Journal 89:57–68.

[16] Holst, J.J., 2007. The physiology of glucagon-like peptide 1. Physiological Reviews 87:1409–1439.

[17] Holst, J.J., Bersani, M., 1991. Assays for peptide products of somatostatin gene expression. Metods Neurosciences 5:3–22.

[18] Jacobsen, S., Olesen, S., Dirksen, C., Jorgensen, N., Bojsen-Moller, K., Kielgast, U., et al., 2012. Changes in gastrointestinal hormone responses, insulin sensitivity, and beta-cell function within 2 Weeks after gastric bypass in non-diabetic subjects. Obesity Surgery 22:1084–1096.

[19] Katsuma, S., Hirasawa, A., Tsujimoto, G., 2005. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochemical and Biophysical Research Communications 329:386–390.

[20] Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., et al., 2003. A G protein-coupled receptor responsive to bile acids. Journal of Biological Chemistry 278:9435–9440.

[21] Kissow, H., Hartmann, B., Holst, J.J., Viby, N.E., Hansen, L.S., Rosenkilde, M.M., et al., 2012. Glucagon-like peptide-1 (GLP-1) receptor agonism or DPP-4 inhibition does not accelerate neoplasia in carcinogen treated mice. Regulatory Peptides 179:91–100.

[22] Kuhre, R.E., Albrechtsen, N.W., Windelov, J.A., Svendsen, B., Hartmann, B., Holst, J.J., 2014. GLP-1 amidation efficiency along the length of the intestine in mice, rats and pigs and in GLP-1 secreting cell lines. Peptides 55:52–57.

[23] Kuhre, R.E., Bechmann, L.E., Wewer Albrechtsen, N.J., Hartmann, B., Holst, J.J., 2015. Glucose stimulates neurotensin secretion from the rat small intestine by mechanisms involving SGLT1 and GLUT2, leading to cell depolarization and calcium influx. American Journal of Physiology Endocrinology and Metabolism 308:E1123–E1130.

[24] Kuhre, R.E., Frost, C.R., Svendsen, B., Holst, J.J., 2014. Molecular mechanisms of glucose-stimulated GLP-1 secretion from perfused rat small intestine. Diabetes 64:370.

[25] Kuhre, R.E., Wewer Albrechtsen, N.J., Deacon, C., Balk-Moller, E., Rehfeld, J.F., Reimann, F., et al., 2016. Peptide production and secretion in GLUTag, NCI-H716 and STC-1 cells: a comparison to native L-cells. Journal of Molecular Endocrinology 56:11.

[26] Kumar, D.P., Asgharpour, A., Mirshahi, F., Park, S.H., Liu, S., Imai, Y., et al., 2016. Activation of transmembrane bile acid receptor TGR5 modulates pancreatic islet alpha cells to promote glucose homeostasis. Journal of Biological Chemistry 291:6626–6640.

[27] Kumar, D.P., Rajagopal, S., Mahavadi, S., Mirshahi, F., Grider, J.R., Murthy, K.S., et al., 2012. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic beta cells. Biochemical and Biophysical Research Communications 427:600–605.

[28] Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., et al., 1999. Identification of a nuclear receptor for bile acids. Science (New York, NY) 284:1362–1365.

[29] Meier, J.J., Nauck, M.A., 2005. Glucagon-like peptide 1(GLP-1) in biology and pathology. Diabetes/Metabolism Research and Reviews 21:91–117.

[30] Nielsen, S., Svane, M.S., Kuhre, R.E., Clausen, T.R., Kristiansen, V.B., Rehfeld, J.F., et al., 2017. Chenodeoxycholic acid stimulates glucagon-like peptide-1 secretion in patients after Roux-en-Y gastric bypass. Physiological Reports 5.

[31] Orskov, C., Jeppesen, J., Madsbad, S., Holst, J.J., 1991. Proglucagon products in plasma of noninsulin-dependent diabetics and nondiabetic controls in the fasting state and after oral glucose and intravenous arginine. The Journal of Clinical Investigation 87:415–423.

[32] Orskov, C., Rabenhoj, L., Wettergren, A., Kofod, H., Holst, J.J., 1994. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. Diabetes 43:535–539.

[33] Parker, H., Adriaenssens, A., Rogers, G., Richards, P., Koepsell, H., Reimann, F., et al., 2012. Predominant role of active versus facilitative glucose transport for glucagon-like peptide-1 secretion. Diabetologia 55: 2445–2455.

[34] Parker, H.E., Wallis, K., le Roux, C.W., Wong, K.Y., Reimann, F., Gribble, F.M., 2012. Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion. British Journal of Pharmacology 165:414–423.

[35] Plaisancie, P., Dumoulin, V., Chayvialle, J.A., Cuber, J.C., 1996. Luminal peptide YY-releasing factors in the isolated vascularly perfused rat colon. Journal of Endocrinology 151:421–429.

[36] Reimann, F., Habib, A.M., Tolhurst, G., Parker, H.E., Rogers, G.J., Gribble, F.M., 2008. Glucose sensing in L cells: a primary cell study. Cell Metabolism 8:532–539.

[37] Röder, P.V., Geillinger, K.E., Zietek, T.S., Thorens, B., Koepsell, H., Daniel, H., 2014. The role of SGLT1 and GLUT2 in intestinal glucose transport and sensing. PLoS One 9 e89977.

[38] Rohde, U., Sonne, D.P., Christensen, M., Hansen, M., Bronden, A., Torang, S., et al., 2016. Cholecystokinin-induced gallbladder emptying and metformin elicit additive glucagon-like Peptide-1 responses. The Journal of Clinical Endocrinology and Metabolism 101:2076–2083.

[39] Rosenkilde, M.M., Andersen, M.B., Nygaard, R., Frimurer, T.M., Schwartz, T.W., 2007. Activation of the CXCR3 chemokine receptor through anchoring of a small molecule chelator ligand between TM-III, -IV, and -VI. Molecular Pharmacology 71:930–941.

[40] Ryan, K.K., Tremaroli, V., Clemmensen, C., Kovatcheva-Datchary, P., Myronovych, A., Karns, R., et al., 2014. FXR is a molecular target for the effects of vertical sleeve gastrectomy. Nature 509:183–188.

[41] Sjovall, J., 1959. On the concentration of bile acids in the human intestine during absorption. Bile acids and sterioids 74. Acta Physiologica Scandinavica 46:339–345.

[42] Sparre-Ulrich, A.H., Gabe, M.N., Gasbjerg, L.S., Christiansen, C.B., Svendsen, B., Hartmann, B., et al., 2017. GIP(3-30)NH2 is a potent competitive antagonist of the GIP receptor and effectively inhibits GIP-mediated insulin, glucagon, and somatostatin release. Biochemical Pharmacology 131:78–88.

[43] Svendsen, B., Holst, J.J., 2016. Regulation of gut hormone secretion. Studies using isolated perfused intestines. Peptides 77:47–53.

[44] Svendsen, B., Pais, R., Engelstoft, M.S., Milev, N.B., Richards, P., Christiansen, C.B., et al., 2016. GLP1- and GIP-producing cells rarely overlap and differ by bombesin receptor-2 expression and responsiveness. Journal of Endocrinology 228:39–48.

[45] Svendsen, B., Pedersen, J., Albrechtsen, N.J., Hartmann, B., Torang, S., Rehfeld, J.F., et al., 2015. An analysis of cosecretion and coexpression of gut hormones from male rat proximal and distal small intestine. Endocrinology 156:847–857.

[46] Thomas, C., Gioiello, A., Noriega, L., Strehle, A., Oury, J., Rizzo, G., et al., 2009. TGR5-Mediated bile acid sensing controls glucose homeostasis. Cell Metabolism 10:167–177.

[47] Trabelsi, M.S., Daoudi, M., Prawitt, J., Ducastel, S., Touche, V., Sayin, S.I., et al., 2015. Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells. Nature Communications 6:7629.

[48] Ullmer, C., Alvarez Sanchez, R., Sprecher, U., Raab, S., Mattei, P., Dehmlow, H., et al., 2013. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. British Journal of Pharmacology 169:671–684.

[49] Van Deest, B.W., Fordtran, J.S., Morawski, S.G., Wilson, J.D., 1968. Bile salt and micellar fat concentration in proximal small bowel contents of ileectomy patients. The Journal of Clinical Investigation 47:1314–1324.

[50] Wang, H., Chen, J., Hollister, K., Sowers, L.C., Forman, B.M., 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Molecular Cell 3:543–553.

[51] Wewer Albrechtsen, N.J., Albrechtsen, R., Bremholm, L., Svendsen, B., Kuhre, R.E., Poulsen, S.S., et al., 2016. Glucagon-like peptide 1 receptor signaling in acinar cells causes growth-dependent release of pancreatic enzymes. Cell Reports 17:2845–2856.

[52] Wong, V.W., Stange, D.E., Page, M.E., Buczacki, S., Wabik, A., Itami, S., et al., 2012. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. Nature Cell Biology 14:401–408.

